Volume 218, number 2, 266–270 FEB 04849 June 1987

Isolation of a thiol-activated T-kininogenase from the rat submandibular gland

Aydin Barlas, Xiaoxing Gao and Lowell M. Greenbaum

Department of Pharmacology and Toxicology and School of Graduate Studies, Medical College of Georgia, Augusta, GA 30912, USA

Received 21 April 1987

T-kininogenase (T-kgnase) activity has been investigated in tissues of the rat and submandibular glands of the rat, mouse and guinea pig. Both rat and mouse submandibular homogenates showed high T-kgnase activity. The enzyme has been purified 360-fold from rat submandibular gland homogenate supernatant fluid. The enzyme has an apparent molecular mass of 28 kDa and a pH optimum of 8.0 toward T-kininogen. It cleaved T-kininogen in catalytic quantities to release T-kinin (Ile-Ser-bradykinin) and small quantities of bradykinin and an unknown kinin. The activity of the enzyme was increased 10-fold in the presence of thiol groups (dithiothreitol) and inhibited by leupeptin (90%) and to a lesser extent by aprotinin (49%), TLCK (46%) and soybean trypsin inhibitor (27%). Pepstatin and PMSF did not inhibit the enzyme. Studies on substrate specificity, pH optimum and agents which inhibit T-kgnase activity demonstrate that this enzyme is different from plasma and tissue kallikreins, cathepsin D, esterase A and esterase B (other known kininogenases). It is the first thiol-activated kininogenase to be reported.

T-Kinin; T-Kininogen; T-Kininogenase; Thiol activation; Submandibular gland

1. INTRODUCTION

Previous studies from this laboratory have demonstrated that free T-kinin (Ile-Ser-bradykinin) is found in blood and inflammatory fluids in carrageenin- and urate-induced inflammation in the rat [1,2]. Since T-kininogen has been shown to be an acute-phase protein which increases in blood following the inflammatory response [3–5], a survey of tissues was made to identify the presence of T-kininogenase (T-kgnase) enzymes, i.e. enzymes that release T-kinin from T-kininogen. We then purified T-kgnase from the rat submandibular gland which showed very high enzyme activity.

Correspondence address: A. Barlas, Department of Pharmacology and Toxicology and School of Graduate Studies, Medical College of Georgia, Augusta, GA 30912, USA

Abbreviations: TLCK, *n-p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SBTI, soybean trypsin inhibitor; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Animals*

Male Sprague–Dawley rats (200–300 g), male Hartley guinea pigs (250–300 g) and male ICR mice (20–25 g) were used.

2.2. *Materials*

Aprotinin (bovine lung), SBTI, TLCK and PMSF were obtained from Sigma. Leupeptin and pepstatin were obtained from Boehringer Mannheim. Sephadex G-200, DEAE-Sephadex A-50 and CH-Sepharose 4B were obtained from Pharmacia. Dithiothreitol and the other SDS-PAGE

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

chemicals were obtained from Bio-Rad. T-Kininogen was prepared as described by Okamoto and Greenbaum [9].

2.3. *Tissue extract preparation*

The animals were killed in a CO_2 chamber. The excised tissues were blotted, minced, weighed and homogenized in 5 vols cold saline using a Polytron homogenizer. The homogenates were centrifuged at 4°C at 20 000 × *g* for 15 min and the supernatant fluid assayed for T-kgnase activity.

2.4. *T-kgnase assay*

The incubation mixture was prepared with 50 mM Tris-HCl buffer (pH 8.0) containing 5 μg T-kininogen, 20 μl of 30 mM *o*-phenanthroline and when appropriate 10 μl of 20 mM dithiothreitol. 1–10 μl homogenate supernatant fluid (diluted 10-fold in the case of the rat mouse submandibular glands) was added to bring the volume to 200 μl. The reaction proceeded for 30 min at 37°C. The solution was boiled for 10 min and a 100 μl sample was radioimmunoassayed for kinins [1]. In experiments involving inhibitors, the inhibitor was added at a final concentration of 10^{-5} M to the reaction mixture.

One unit of the enzyme is defined as the amount of the enzyme which releases 1 ng T-kinin in 30 min under the above conditions. Specific activity is expressed as U/mg protein.

2.5. *Kallikrein assay*

Kallikrein activity was measured using the fluorogenic peptide substrate Pro-Phe-Arg-MCA according to Kato et al. [6].

2.6. *Purification of T-kgnase from rat submandibular gland*

All purification procedures were carried out at 4°C. During the purification, the activities in the fractions were assayed against two different substrates, T-kininogen and Pro-Phe-Arg-MCA, in order to separate T-kgnase from kallikrein-like enzymes. The 20 000 × *g* supernatant fluid (section 2.3) was recentrifuged at 100 000 × *g* for 60 min. The resultant supernatant fluid was fractioned with solid ammonium sulfate and the precipitate between 30 and 100% saturation was collected. The precipitate was dissolved in distilled water and dialyzed for 24 h against 0.01 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl (0.1 M NaCl has been found to stabilize the purified enzyme). The dialyzate was applied to a column of Sephadex G-200 (1.5 × 30 cm) previously equilibrated with the dialysis buffer. The fractions with T-kgnase activities were pooled and applied to DEAE-Sephadex A-50 column (1.2 × 25 cm) previously equilibrated with the same buffer. After washing the column with 50 ml buffer, the elution was performed by a linear gradient of 150 ml equilibrating buffer against 150 ml of the same buffer with 0.5 M NaCl. The flow rate was 30 ml/h and 3.3-ml volumes were collected. The fractions having T-kgnase activity were pooled, dialyzed against equilibrating buffer, and lyophilized. The leupeptin-agarose affinity column was prepared by coupling 25 mg leupeptin with 4 g CH-Sepharose 4B (Pharmacia) using the carbodiimide coupling method according to Pharmacia. The column (0.9 × 20 cm) was equilibrated with 0.01 M sodium phosphate buffer (pH 7.2) with 0.1 M NaCl. The lyophilizate from the previous step was dissolved in water and the conductivity was adjusted to the equilibrating buffer. After applying the sample and washing the column with 40 ml buffer, the elution was performed with a linear gradient of 30 ml equilibrating buffer against 30 ml of the same buffer with 2 M NaCl. The flow rate was 12 ml/h and 2-ml volumes were collected. The fractions with T-kgnase activity were pooled, dialyzed and lyophilized.

Table 1

Tissue content of T-kgnase

Tissue	Units enzyme/μl homogenate[a]	
	No DTT	DTT
Submandibular gland (rat)	18	75
Submandibular gland (mouse)	28	56
Submandibular gland (guinea pig)	0.05	0.1
Liver (rat)	0.2	0.2
Lung (rat)	0.2	0.4
Heart (rat)	0.2	0.2
Kidney (rat)	0.2	0.4
Pancreas (rat)	0.4	0.6
Spleen (rat)	0.02	0.09

[a]DTT concentration was 10^{-3} M

Protein determination was carried out by the method of Lowry et al. [7], using bovine serum albumin as a standard. SDS-PAGE was performed with 12.5% acrylamide by the method of Laemmli [8]. Reversed-phase HPLC experiments were carried out as in [4].

3. RESULTS

Table 1 demonstrates that all tissues assayed had low levels of T-kgnase activity. The submandibular glands of the rat and mouse had much higher levels. The submandibular homogenates in the presence of dithiothreitol increased their T-kgnase activity 2–5-fold. The guinea pig submandibular gland showed no activity.

We have examined the effect of number of inhibitors on T-kgnase activity of the rat submandibular gland homogenates (table 2). Leupeptin strongly inhibited this activity. Aprotinin, TLCK and SBTI had lesser inhibitory actions. Pepstatin and PMSF had no inhibitory activity.

Table 3 summarizes the steps for purification from the submandibular gland of 10 rats, 7 days post-injection with Freunds complete adjuvant. During the chromatography using DEAE-Sephad-

Table 2

Effect of agents on T-kgnase from rat submandibular gland

Agent	Units of enzyme	% inhibition
None	82	–
Leupeptin	11	87
Aprotinin	42	49
TLCK	45	46
SBTI	60	27
Pepstatin	82	0
PMSF	82	0

Homogenate supernatant fluid was preincubated with 10^{-5} M agent for 15 min at 37°C before incubation with T-kininogen in the presence of 10^{-3} M DTT for 30 min

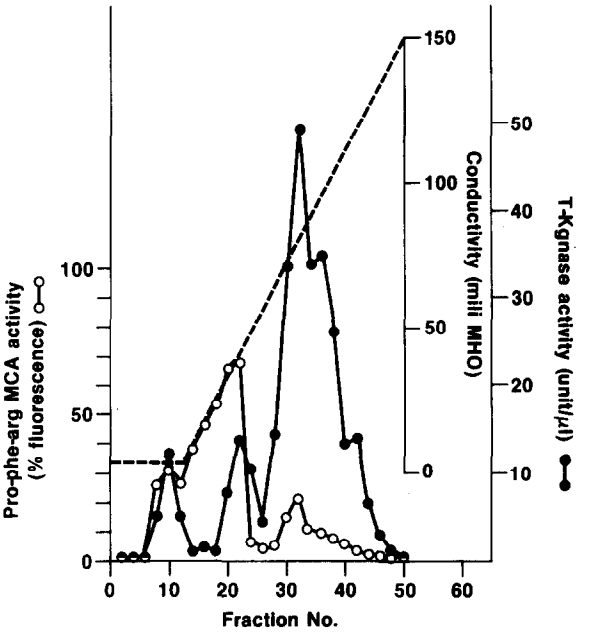

Fig.1. Leupeptin-agarose affinity chromatography. 5 mg protein were loaded onto a column (0.9×20 cm) previously equilibrated with 0.01 M sodium phosphate (pH 7.2) with 0.1 M NaCl. The elution was performed with a linear salt gradient (0.1–2.0 M NaCl); flow rate, 12 ml/h; 2 ml per fraction collected. (○—○) Kallikrein activity, (●—●) T-kgnase.

Table 3

Purification of T-kgnase from rat submandibular gland

Treatment	[a]Units/ml ($\times 10^{-3}$)	Total/units ($\times 10^{-3}$)	Protein (mg/ml)	Specific activity ($\times 10^{-3}$)	Purification (-fold)	Yield (%)
100 000 × *g* supernatant fluid	75	1200	11.9	6	1.0	100
Ammonium sulfate (30–100%)	70	1131	8.8	8	1.3	94
Sephadex G-200	44	1630	2.6	17	2.7	86
DEAE-Sephadex A-50	32	518	0.34	95	15.0	43
Leupeptin-agarose	26	436	0.01	2184	360	35

[a]One unit of enzyme is the amount of the enzyme which releases 1 ng T-kinin in 30 min at 37°C

ex A-50, T-kgnase activity was eluted as a broad peak at a conductivity of 8 $m\Omega^{-1}$ (not shown). Pooled fractions (50–80) from DEAE-Sephadex A-50 were applied to the leupeptin-agarose affinity column. In this chromatography, most of the T-kgnase activity was eluted with high salt concentration (fig.1). Those fractions with high T-kgnase activity showed almost no Pro-Phe-Arg-MCA activity, and reached a 360-fold purification from the original supernatant. All further characterization was performed with pooled material eluted from the leupeptin-agarose affinity column. SDS-PAGE (fig.2) gave a single band with non-reduced T-kgnase with an apparent molecular mass of 28 kDa. However, SDS-PAGE of T-kgnase reduced with mercaptoethanol gave two bands of 22 and 6 kDa. These data suggest that T-kgnase is composed of a heavy chain (22 kDa) and a light chain (6 kDa) linked together through a disulfide bridge.

Fig.3 shows reversed-phase HPLC of kinins released from T-kininogen by T-kgnase. This experiment indicates that T-kinin was the major kinin released. A small amount of bradykinin and

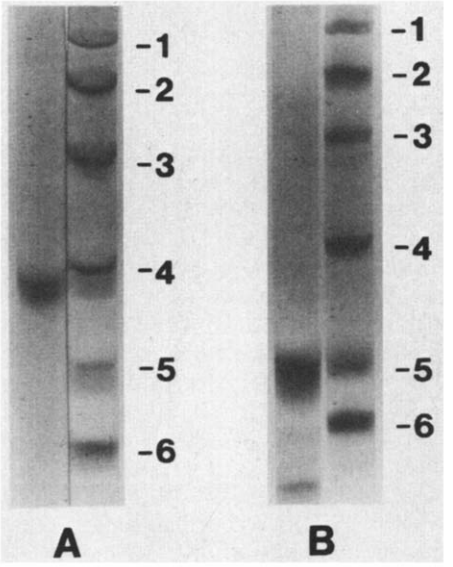

Fig.2. SDS-PAGE of T-kgnase. (A) Non-reduced gel, (B) reduced gel. Molecular mass standards: (1) phosphorylase *b* (92.5 kDa); (2) bovine serum albumin (67.0 kDa); (3) ovalbumin (45.0 kDa); (4) carbonic anhydrase (31.0 kDa); (5) soybean trypsin inhibitor (21.5 kDa); (6) lysozyme kDa (14.0 kDa). All stained with Coomassie brilliant blue G-250.

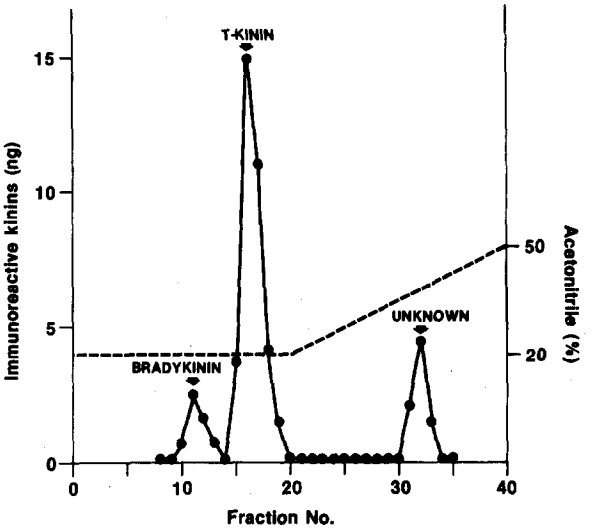

Fig.3. Reversed-phase HPLC of kinins released from T-kininogen by T-kgnase. 30 ng kinins was injected into the column (ODS; 4.6×25 cm). Kinins were eluted with a solvent system of 0.04 M triethylammonium formate buffer (pH 4.0)/acetonitrile (80:20) for the first 40 min, then with a linear gradient of acetonitrile (20–50%) for the last 40 min. Flow rate was 0.75 ml/min; quantitation was by radioimmunoassay.

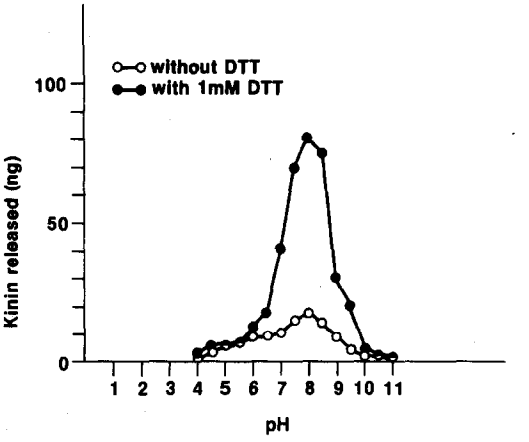

Fig.4. Effects of dithiothreitol and pH on T-kgnase activity. 0.2 μg purified T-kgnase was incubated with 5 μg T-kininogen for 30 min at 37°C in the absence (○—○) and presence (●—●) of 1 mM dithiothreitol at different pH values. 50 mM sodium acetate for pH 4–6; 50 mM Tris-HCl for pH 7–8; 50 mM sodium bicarbonate for pH 9–11.

unknown kinin was also observed.

Studies showed that T-kgnase had a pH optimum of 8.0. In the presence of 1 mM dithiothreitol its activity increased about 10-fold (fig.4).

4. DISCUSSION

A number of enzymes have been reported to release T-kinin (Ile-Ser-bradykinin) from T-kininogen. Okamoto and Greenbaum [10,11] were the first to demonstrate that trypsin releases T-kinin from T-kininogen in rat blood. They also demonstrated that cathepsin D released T-kinin from T-kininogen at acidic pH. A cathepsin E-like protease and 72 kDa proteinase have been reported to release T-kinin from T-kininogen by a consecutive cleavage of the protein [12]. An acidic protease of Murphy-Sturm lymphosarcoma has been reported to release T-kinin [13]. In addition to these kininogenases, the submandibular gland of the rat contains a variety of proteases which have kininogenase activity; these include kallikrein [14], tonin [15], esterase A [16] and B [17]. The thiol-activated T-kininogenase as reported here is unique and termed by us 'T-kgnase'. From the substrate specificity, pH optimum and response to inhibitors, T-kgnase differs from other known kininogenases. In this regard, it is of interest that T-kininogen is known to be a thiol protease inhibitor [18]. The current findings would indicate that it binds to thiol proteases and in the case of T-kgnase acts as a substrate for this enzyme. Moreau et al. [19] have made the claim that T-kininogen is not a true kininogen since trypsin is required in stoichiometric amounts to release T-kinin. The current report clearly demonstrates that T-kgnase, present in submandibular glands of the rat and mouse, can utilize T-kininogen as a substrate. The enzyme acts in catalytic quantities in the presence of thiol groups. The conclusion is reached that T-kininogen is a substrate for the release of the vasoactive mediator T-kinin. This is of special importance since it is an acute-phase protein [4,20]. Thiol groups may serve as a regulator of T-kgnase activity and T-kinin release from T-kininogen.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Jannie Jones for technical assistance. This investigation was supported by NIH grant HL-32183.

REFERENCES

[1] Barlas, A., Sugio, K. and Greenbaum, L.M. (1985) FEBS Lett. 190, 268–270.
[2] Sugio, K., Greenbaum, L.M. and Barlas, A. (1986) Fed. Proc. 45, 3039.
[3] Barlas, A., Greenbaum, L.M. and Okamoto, H. (1984) Fed. Proc. 43, 2150.
[4] Barlas, A., Okamoto, H. and Greenbaum, L.M. (1985) Biochem. Biophys. Res. Commun. 129, 280–286.
[5] Kageyama, R., Kitamura, N., Ohkuba, H. and Nakanishi, S. (1985) J. Biol. Chem. 260, 12060–12064.
[6] Kato, H., Adachi, N., Iwanaga, S., Abe, K., Takada, K., Kimura, T. and Sakakibara, S. (1980) J. Biochem. 87, 1127–1132.
[7] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biochem. Chem. 193, 265–275.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Okamoto, H. and Greenbaum, L.M. (1986) Adv. Exp. Med. Biol. 198A, 69–76.
[10] Okamoto, H. and Greenbaum, L.M. (1983) Life Sci. 32, 2007–2013.
[11] Okamoto, H. and Greenbaum, L.M. (1983) Biochem. Biophys. Res. Commun. 112, 701–708.
[12] Sakamoto, W., Yoshikawa, K., Yokoyama, A. and Kohri, M. (1986) Biochim. Biophys. Acta 884, 607–609.
[13] Bedi, G.S., Balwierczak, J. and Back, N. (1983) Biochem. Biophys. Res. Commun. 112, 621–628.
[14] Shachter, M. (1979) Pharmacol. Rev. 31, 1–18.
[15] Ikeda, M. and Arakawa, K. (1984) Hypertension 6, 222–228.
[16] Chao, J. (1983) J. Biol. Chem. 258, 4434–4439.
[17] Khullar, M., Scicli, G., Carretero, O.A. and Scicli, A.G. (1986) Biochemistry 25, 1851–1857.
[18] Sueyoshi, T., Enjyoji, K., Shimada, T., Kato, H., Iwanaga, S., Bando, Y., Kominami, E. and Katunuma, N. (1985) FEBS Lett. 182, 193–195.
[19] Moreau, M., Gutman, N., El Moujahed, A., Esnard, F. and Gauthier, F. (1986) Eur. J. Biochem. 159, 341–346.
[20] Cole, T., Inglis, A.S., Roxburgh, C.M., Howlett, G.J. and Schrieber, G. (1985) FEBS Lett. 182, 57–61.